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THE EFFECT OF COLCHICINE ON MIGRATION OF PIGMENT GRANULES IN THE MELANOPHORES OF *FUNDULUS HETEROCLITUS*[1]

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Previous work has shown that colchicine has an effect on the movement of melanin granules in the melanophores of isolated frog skin. Thus pretreatment of frog skin with colchicine enhances the darkening produced by melanocyte-stimulating hormone (MSH) (Malawista, 1965) and inhibits the lightening which follows upon the removal of MSH (Wright, 1955; Malawista, 1965). Colchicine has also been found to inhibit the aggregation of pigment in tissue cultured melanophores of *Xenopus laevis* after removal of MSH (Kulemann, 1960). The proposal has been advanced that in frog melanophores this drug has an effect on cytoplasmic viscosity (Malawista, 1965; Malawista, Asterita, and Marsland, 1966).

Recently Bikle, Tilney and Porter (1966) have shown that an ordered array of microtubules is present in the branching processes of the melanophores of the killifish, *Fundulus heteroclitus*. These observations have been confirmed by Novales and Novales (1966) and Green (1968). Bikle *et al.* (1966) suggested that these microtubules may be involved in the movement of the pigment granules.

Colchicine has been demonstrated to have an effect on cytoplasmic microtubules. This alkaloid brings about disruption of mitotic spindle microtubules in a variety of dividing cells (de Harven and Bernhard, 1956; Robbins and Gonatas, 1964; Pickett-Heaps, 1967). Colchicine also brings about disassembly of microtubules in a number of interphase cells. These include HeLa cells (Robbins and Gonatas, 1964; Freed, Bhisey, and Lebowitz, 1968), axopods of *Actinosphaerium* (Tilney, 1965), human lymphocytes (Malawista and Bensch, 1967), and human platelets (White, 1968). The mechanism by which colchicine breaks down microtubules is unknown. The effect of this drug on the mitotic apparatus has been attributed to a possible combination with sulfhydryl groups of the spindle proteins (Dustin, 1949; Galzigna, 1961). Reaction of colchicine with thiol groups is also indicated by studies on chick embryos in which the inhibitory effect of colchicine on embryonic induction is completely reversed by cysteine hydrochloride (Diwan, 1966). In view of the known effect of colchicine on microtubules, a study was undertaken to test the effects of this drug on pigment migration in *Fundulus* melanophores. In addition, in order to gain further insight into the mechanism of action of colchicine, work was carried out to see if its effect could be reversed by cysteine

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hydrochloride. The action of colchicine was studied in relation to its effect on the response of the melanophores to epinephrine. Epinephrine was used because it produces rapid aggregation of the melanin in *Fundulus* melanophores (Spaeth and Barbour, 1917) and has been shown to act directly on the fish melanophore (Fujii, 1961).

Materials and Methods

Experiments were performed with isolated scales from mature specimens of the killifish, *Fundulus heteroclitus*. The fishes were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. They were kept in an "Instant Ocean" Culture System in artificial sea water which was maintained between 10 and 13° C. For each experiment scales were removed with fine forceps from the anterior dorsal and dorsolateral regions of the fish. The scales were mounted on a glass coverslip which was sealed to a Dick perfusion chamber (Dick, 1955). The scales were mounted and the chamber was sealed with Vaspar (4 parts beeswax to 1 part yellow vaseline). Ringer's solution was perfused through the chamber.

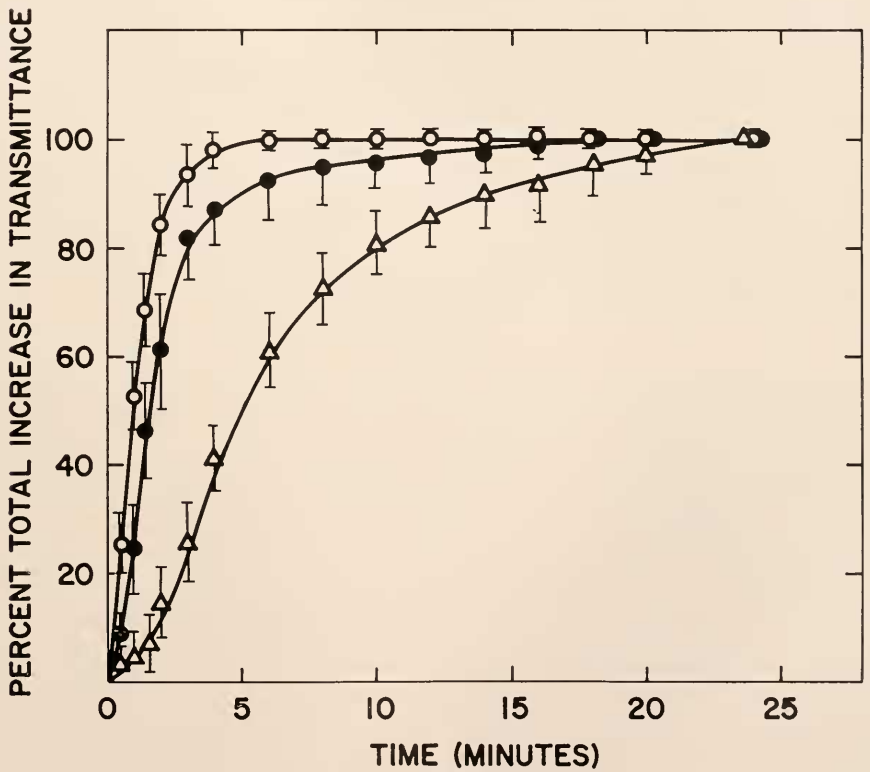

Figure 1. The effects of colchicine pretreatment for 40 minutes on the response of melanophores to 0.06 m*M* epinphrine. Points are means ± standard errors. (○) Ringer, 18 scales; (●) 0.5 m*M* colchicine, 8 scales; (△) 5 m*M* colchicine, 10 scales.

A scale remained in Ringer until the melanin granules in the melanophores were dispersed (15 to 30 minutes). The test solutions were then perfused through the chamber.

All test substances were freshly prepared for each experiment. Each was dissolved in frog Ringer's solution, which had the following composition in g per l of distilled water: NaCl, 6.50; KCl, 0.14; $CaCl_2 \cdot 2H_2O$, 0.16; $NaHCO_3$, 0.20. The pH of the solutions was between 7.0 and 7.4. Colchicine (Nutritional Biochemicals Corp.) was prepared at 5 m*M* and 0.5 m*M* concentrations and protected from light before use. A 5 m*M* and 50 m*M* solution of cysteine hydrochloride (Nutritional Biochemicals Corp.) was made up in Ringer buffered, respectively, at pH 7.4 with 5 m*M* and 10 m*M* Tris-HCl buffer. The pH of both solutions was adjusted back to neutrality with a few drops of 0.10 N NaOH. Epinephrine was prepared by dilution of 1:1000 adrenalin chloride (Parke, Davis and Co.) with Ringer to give a 0.06 m*M* solution. The experiments were run at room temperature, between 22 and 24° C.

Changes in the state of melanin dispersion in the melanophores were measured by a photoelectric method (Hill, Parkinson and Solandt, 1935; Smith, 1936; Fujii, 1959). Light transmitted through the scale was directed onto the search unit of a Photovolt Multiplier Photometer, model 520-M. This unit replaced one ocular of a Spencer binocular light microscope. Aggregation of the pigment was measured as an increase in per cent transmittance. The response of the melanophores to a mixture of colchicine and epinephrine was first studied, after which the effect of pretreatment in colchicine on the response to epinephrine was tested. The effect of colchicine on punctate epinephrine-treated melanophores was also studied. The degree of expansion was measured in ocular micrometer units. This expansion, expressed as a percentage of the original response, was based on the difference between the diameter of the melanophore in the fully aggregated state and the greatest diameter in the fully dispersed condition. The ability of cysteine hydrochloride to reverse the effect of colchicine was finally tested.

Results

A 0.06 m*M* solution of epinephrine was found to be the minimum concentration which produced consistent rapid aggregation of the melanin. With a mixture of colchicine and epinephrine, only a slight decrease in the rate of aggregation of the pigment occurred as compared to a combination of Ringer and epinephrine. Thus, pretreatment in colchicine was carried out. Pretreatment produced a decrease in the rate of melanin aggregation in response to epinephrine as compared to the untreated scales. The rate of response depended upon the length of pretreatment and the concentration of colchicine. Thus 40 minutes pretreatment in 0.5 m*M* colchicine had only a slight effect on the rate of pigment aggregation (Fig. 1). However, 5 m*M* colchicine had a significant effect (Fig. 1). By the end of 6 minutes the difference in per cent aggregation was significant at the 5% level. When the pretreatment time was increased to 90 minutes, the response to epinephrine was greatly decreased (Fig. 2). Pigment in melanophores pretreated in Ringer was aggregated within 6 minutes, whereas in those pretreated with colchicine the pigment took 20 to 30 minutes to aggregate. The effects of colchicine could not be "washed out," as shown by the following. Five scales were pretreated

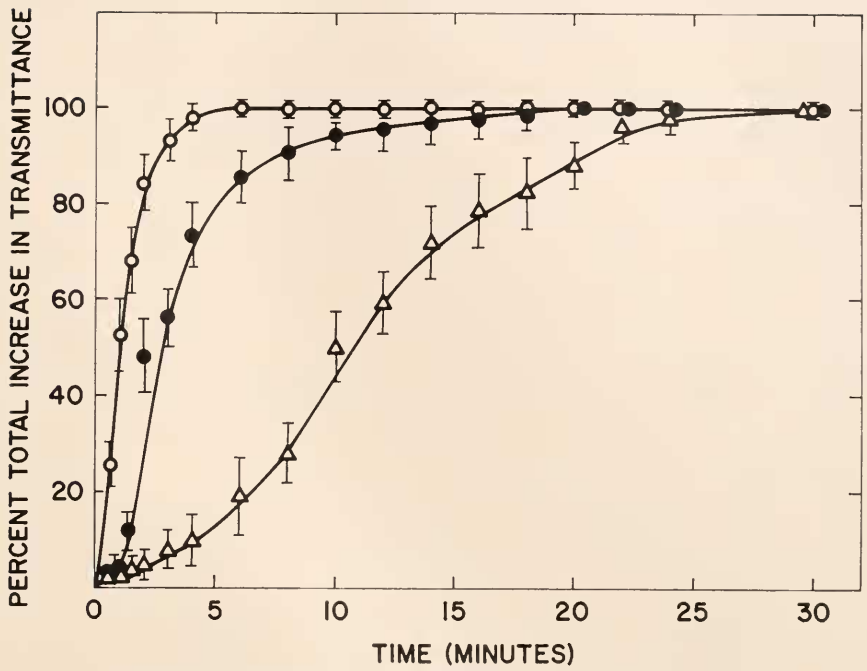

FIGURE 2. The effects of colchicine pretreatment for 90 minutes on the response of melanophores to 0.06 m*M* epinephrine. Points are means ± standard errors. (○) Ringer, 18 scales; (●) 0.5 m*M* colchicine, 8 scales; (△) 5 m*M* colchicine, 10 scales.

in 5 m*M* colchicine for 60 minutes. The scales were then perfused with several changes of Ringer for 60 minutes. When epinephrine was added the results were the same as those obtained from the unwashed scales.

Colchicine also produced dispersion of pigment in punctate epinephrine-treated melanophores. A 5 m*M* solution of colchicine brought about a dispersion of pigment granules which was significantly greater than that produced by Ringer alone (Fig. 3). By the end of 60 minutes the difference was significant at the 5% level. Enhanced dispersion was observed after 80 minutes treatment in colchicine.

Incubation in 50 m*M* cysteine hydrochloride alone following a 60 minute pretreatment with 5 m*M* colchicine had no effect on the melanophores. However, the effects of colchicine on the response to epinephrine were reversed by cysteine (Fig. 4). This reversal depended upon the concentration of cysteine used. Thus, 5 m*M* cysteine did not have a significant effect on colchicine action, whereas, treatment in 50 m*M* cysteine resulted in a normal response to epinephrine. Cysteine by itself had no effect on dispersion of pigment. A 50 m*M* solution had the same effect as Ringer on punctate epinephrine-treated melanophores. Pretreatment with cysteine also did not affect dispersion produced by colchicine. Increasing the pretreatment in 10 m*M* cysteine from 10 to 20 minutes produced only a slight decrease in the dispersion response. Increasing the concentration of cysteine to 50 m*M* slightly enhanced the initial dispersion produced by colchicine.

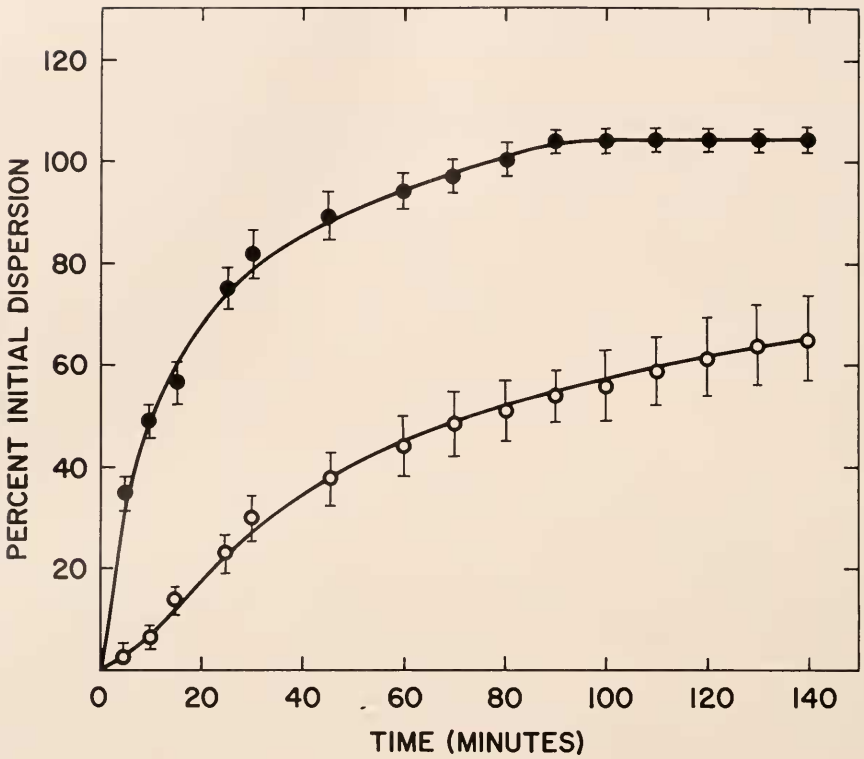

FIGURE 3. Response of punctate epinephrine-treated melanophores to colchicine. Points are means ± standard errors. (○) Ringer, 24 scales; (●) 5 m*M* colchichine, 21 scales.

DISCUSSION

Colchicine decreased the rate of pigment aggregation in *Fundulus* melanophores in response to epinephrine. The action of this drug depended upon its concentration and pretreatment time (Figs. 1 and 2). The concentrations of colchicine used here are within the ranges that have been used on various cell types without affecting their viability. These include cultured fibroblasts from chick embryo heart (Miszurski, 1949), onion root tip (Sedar and Wilson, 1951) and sea urchin gastrulae (Tilney and Gibbons, 1968). In the present study, the effects produced by colchicine are similar to those observed by Malawista (1965) on dermal melanophores in frog skin. Malawista found that colchicine produced partial inhibition of lightening in skins in response to epinephrine. In the present work, colchicine also brought about dispersion of pigment granules in punctate melanophores which was greater than that produced by Ringer's solution alone. Again, this is in accord with Malawista's results wherein he observed that colchicine produced a darkening of lightened frog skins.

There are several possibilities as to the mode of action of colchicine. This alkaloid might affect the viscosity of the cytoplasm bringing about solation and inhibiting the gelation which is necessary for or which accompanies aggregation of

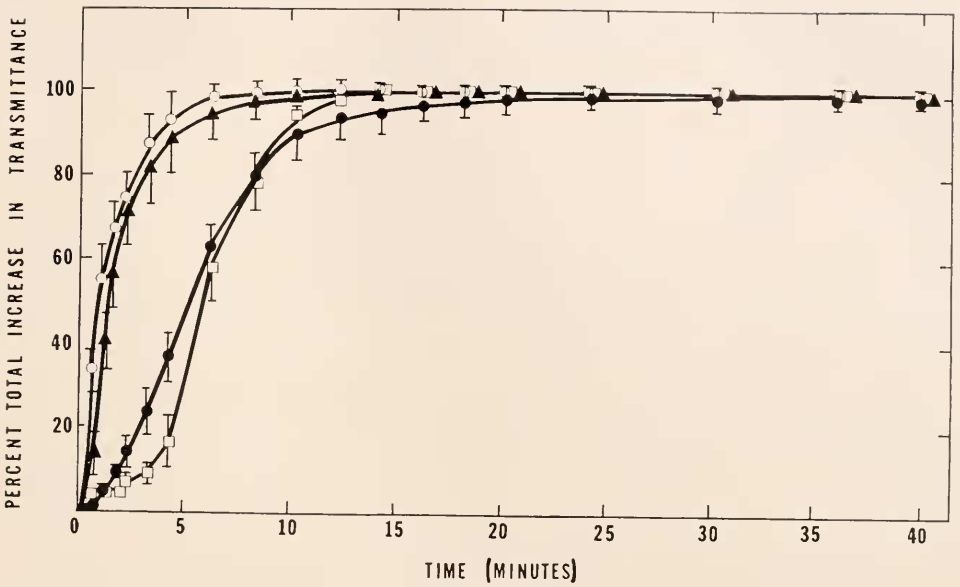

FIGURE 4. Effects of cysteine hydrochloride on the response of colchicine-treated melanophores to 0.06 m*M* epinephrine. Points are means ± standard errors. Pretreatment for 60 minutes in (○) Ringer, 16 scales; (●) 5 m*M* colchicine, 12 scales; (□) 5 m*M* colchicine plus 30 minutes in 5 m*M* cysteine, 5 scales; (▲) 5 m*M* colchicine plus 30 minutes in 50 m*M* cysteine, 13 scales.

the pigment. Matthews (1931) and Marsland (1944) have presented evidence for the view that cytoplasmic solation occurs during melanin dispersion and that melanin aggregation is associated with gelation. Colchicine is known to decrease cytoplasmic viscosity in sea urchin eggs (Beams and Evans, 1940), grasshopper neuroblasts (Gaulden and Carlson, 1951), tissue cultured rat striated muscle fibers (Godman and Murray, 1953; Godman, 1955) and cultured rat fibroblasts (Murray, deLam, and Chargaff, 1955). Malawista (1965) has proposed a general model of colchicine action on gelated cytoplasmic systems. In this model, colchicine, in a gradual, dosage-dependent manner lowers the potential limit of gelation and thus interferes with the maintenance of the gelated state. It is thus possible that in *Fundulus* melanophores colchicine has some effect on the sol-to-gel equilibrium, interfering with the development or maintenance of the gelated condition.

The action of colchicine has also been attributed to a disassembly of cytoplasmic microtubules. This alkaloid appears to disrupt the microtubules by binding to sites of interaction between the protein subunits (Taylor, 1965; Borisy and Taylor, 1967a, 1967b; Shelanski and Taylor, 1967). Dissolution of microtubules by colchicine has been found to be correlated with cessation of particle movement. Tilney (1965) observed that under the influence of colchicine particle streaming within the axopods of a heliozian ceases and coincidently the microtubules disassemble. Freed (1965) also found that treatment of HeLa cells with colchicine led to an inhibition of particle movement and a disappearance of microtubules (Freed, Bhisey, and Lebowitz, 1968). In *Fundulus* melanophores, microtubules

run parallel to the longitudinal axis of the cell processes (Bikle, Tilney, and Porter, 1966; Green, 1968). The melanin granules in these extensions are aligned in columns and appear to move parallel to the microtubules along relatively fixed channels. As in other cell types these microtubules are arranged in such a way as to give support and thus serve as cytoskeletal elements, to guide intracellular migration of cytoplasmic particles, and to possibly exert a motive force on particle movement (Sandborn, Koen, McNabb, and Moore, 1964). However, the precise role of these tubular structures is unknown. It is possible that in *Fundulus* melanophores colchicine is acting upon the microtubules and in turn influencing pigment migration. Since colchicine decreases the rate of pigment aggregation but not dispersion, this indicates that microtubules, if involved, influence only the aggregation of the pigment granules.

The effects of colchicine are similar to those produced by high hydrostatic pressure. High hydrostatic pressure produces dispersion and inhibits aggregation of pigment granules in *Fundulus* melanophores (Marsland, 1944; Marsland and Meisner, 1967). It also causes breakdown of the microtubules of the mitotic apparatus (Zimmerman and Marsland, 1964) and of heliozoan axopodia (Tilney, Hiramoto, and Marsland, 1966). This evidence thus indicates that colchicine and high hydrostatic pressure produce similar changes. However, it is unknown whether these agents produce their effect by acting on cytoplasmic viscosity, microtubules, or both.

Heavy water (D_2O) antagonizes the effect of high hydrostatic pressure on *Fundulus* melanophores, possibly through stabilization of microtubules or cytoplasmic gelation (Marsland and Meisner, 1967). The effects of colchicine are opposite to those produced by D_2O. Studies indicate that D_2O stabilizes the microtubules of the mitotic appartus (Marsland and Zimmerman, 1965) and of heliozoan axopodia (Marsland and Hirshfield, 1968), whereas colchicine breaks down these structures. Low concentrations of D_2O oppose the anti-mitotic effects of colchicine on the mitotic spindle of sea urchin eggs (Marsland and Hecht, 1968). Thus it would be of interest to see if D_2O opposes the action of colchicine on melanophores, in particular, the action of colchicine on the response to epinephrine.

It is also possible that colchicine could be interfering in some manner in one of the steps coupling epinephrine action to melanin aggregation, perhaps at the level of the epinephrine receptors. However, there is no evidence for this view or reason to believe that it is acting in this manner.

Cysteine hydrochloride by itself had no direct effect on pigment migration. It did not produce aggregation of pigment of colchicine-treated melanophores nor did it increase the rate of dispersion beyond that produced by Ringer, in punctate melanophores. Shizume, Lerner and Fitzpatrick (1954) also found that cysteine had no direct effect on frog melanophores. However, cysteine reverses the effect of colchicine on the response of melanophores to epinephrine (Fig. 4). This indicates that the action of colchicine involves a reaction with sulfhydryl groups. Work in other systems points to a similar action for colchicine. For example, the inhibitory effect of colchicine on induction in chick embryos is also reversed by cysteine hydrochloride, thus suggesting a reaction of colchicine with sulfhydryl groups (Diwan, 1966). Work on the mitotic spindle also indicates that colchicine may exert its antimitotic action by reversible oxidation of thiolic proteins (Dustin, 1949;

Galzigna, 1961). On the other hand, the dispersing effect of colchicine was not reversed by cysteine. This may indicate that the action of colchicine on dispersion does not depend on a tying up of SH groups.

Summary

1. The effects of colchicine were studied on pigment migration in melanophores of the killifish, *Fundulus heteroclitus*.

2. Pretreatment of the melanophores in colchicine resulted in a decrease in the rate of melanin aggregation in response to epinephrine. The rate of response depended upon the length of pretreatment and the concentration of colchicine.

3. Colchicine produced dispersion of pigment in punctate epinephrine-treated melanophores which was greater than that produced by Ringer alone.

4. Cysteine reversed the effect of colchicine on the response of melanophores to epinephrine. This reversal depended upon the concentration of cysteine used.

5. The mechanism of action of colchicine is unclear but is discussed in relation to possible effects on cytoplasmic viscosity and microtubules.

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